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Effects of a Gonadotropin-Releasing Hormone Analog (HOE 766) on Germinal and Interstitial Compartments during the Annual Cycle in the Green Frog: *Rana esculenta*

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ABSTRACT—Seasonal changes, related to treatments carried out using a GnRH-analog (HOE766, GnRHA), germinal and interstitial compartment activites were studied in the frog, *Rana esculenta* testis. We have investigated on: a) annual variations of mitotic index (MI) of primary spermatogonia (I SPG), b) changes of interstitial area, c) fluctuation of testicular androgen concentration, and d) variations of sperm releasing activity during different periods of the year were also studied. Our results indicate that testes of *Rana esculenta* show a cycle of responsiveness to GnRHA treatments with respect to androgen production, interstitial growth, I SPG multiplication and spermiation. For each phenomenon considered, the extent of the response is peculiar of the period of the year in which the experiment is carried out.

INTRODUCTION

Seasonal gonadal activity has been extensively studied in vertebrates [1] with respect to the regulation of steroidogenesis and spermatogenesis. The hypothalamus-hypophyseal control mechanisms appear to play a central role in driving the cascade of events which turns on the breeding season. Indeed, environmental cues (light and temperature) act either modulating the release of GnRH from hypothalamus or directly controlling the pituitary sensitivity in term of gonadotropin discharge; this, in turn, is further regulated by the steroid milieu [2, 3]. Also factors inherent to the testis have been claimed to contribute to the phenomenon determining a cycle of gonadal sensitivity to gonadotropin stimulation [4].

In the frog, *Rana esculenta,* interstitial and germinal compartments show different seasonality since steroidogenesis and spermatogenic wave occur at high rate during late autumn-early spring and late spring-early autumn, respectively [2]. Therefore, it is interesting to have insight in the physiology of the two different testicular compartments in this frog species which may constitute an useful animal model to investigate the regulation of testicular functions.

Scope of the present paper is to study changes of the responsiveness of the pituitary-testis axis during the annual cycle, treating intact frogs, *Rana esculenta,* with a gonadotropin-releasing hormone analog (HOE 766). Indeed, a divergent response elicited by the treatment on the endocrine and germinal tissue respectively, may indicate the existence of local mechanisms which activate testicular compartments to be responsive to the same stimulus separately in different periods of the year.

MATERIALS AND METHODS

Adult frog, *Rana esculenta,* were collected during 1987, from January to December. Each month, 10 freshly collected males received 900 ng GnRH agonist, (GnRHA HOE 766 Hoechst, 45 ng/g BW) in Krebs-Ringer bicarbonate buffer (KRB). Injections were given into the dorsal sac on alternate days for 2 weeks. Simultaneously, 10

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animals were used as controls and injected with vehicle (KRB) alone. During March, June and October 5 animals per experimental group were decapitated 2.5 h after the last injection and right testes were used to determine the androgen concentration by RIA as described previously [4, 5]. Since the antiserum used was cospecific for testosterone and 5 α-dihydrotestosterone, the results are expressed as "androgens". Intra- and interassay coefficients of variations were 6% and 9%, respectively and sensitivity was 2 pg/tube. The left testes were fixed in Bouin's fluid and examined, after staining with hemallum and eosin, for interstitial area calculation and for signs of spermiation. These periods were chosen since they are peculiar of the seasonal cycle of *Rana esculenta*. Indeed, during March androgens are at the highest concentration and spermatogenesis begins, reaching its maximum during late spring-early summer (June). In October, the spermatogenic wave is near to be extinguished and androgen levels start to increase again [2, 4, 6–8]. Interstitial area was measured in 10 randomly chosen sections per experimental group and expressed in μm^2 (mean $\pm$ SD). Spermiation was calculated as the number of empty tubules per 100 total tubules observed in randomly chosen sections.

The remaining animals collected all year around received 100 μg colchicine (Prolabo, Paris) into the dorsal sac and were decapitated 24 h later. Testes were fixed in Bouin's fluid and 5–6 μm cross-sections were stained with hemallum and eosin. Mitotic index (MI) of the primary spermatogonia (I SPG) was expressed as number of metaphases per total I SPG counted multiplied by 100 [9].

Significance of differences was evaluated using Student's "*t*" test for between group comparisons and Duncan's test for multigroup comparisons. "Chi square" test was also carried out when appropriate.

RESULTS

a) *Annual variation of MI of I SPG in GnRHA-treated frogs*

In control animals the MI of I SPG showed two

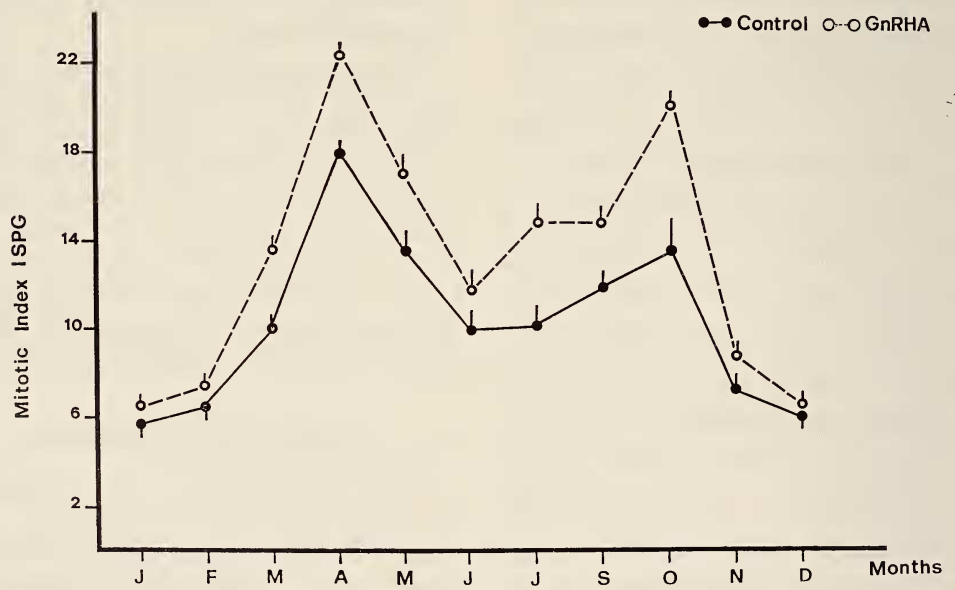

Fig. 1. Annual variations of ISPG mitotic index in vehicle and GnRHA treated frogs, *Rana esculenta*. Duncan's test was used to evaluate significance of differences among vehicle or GnRHA-injected animals. Student's "*t*" test was used to evaluate significance of differences between vehicle and GnRHA-injected animals of the same month. Values are the mean $\pm$ SD.

peaks of proliferative activity (Fig. 1). Indeed, I SPG mitosis peaked in April ($P<0.001$ vs February or June) and October ($P<0.001$ vs July and November), although during October the peak of proliferative activity was less pronounced ($P<0.001$ vs April). Similarly, GnRHA treated frogs showed two significant peaks of proliferative activity of I SPG ($P<0.001$) simultaneously with those of control animals. Moreover, in all periods of the year GnRHA stimulated significantly I SPG multiplication ($P<0.05$ at least). Interestingly, the extent of stimulation observed from June until October was greater ($P<0.01$) as compared with the remaining periods. Indeed, while GnRHA increased the MI of I SPG 127.32% ±11.2 during June-October period, only 116.16% ±6.4 was the stimulation measured in the other months.

b) *Measurement of testicular androgen concentration and interstitial area in different periods of the year*

Testicular androgen content (Fig. 2) was highly stimulated by GnRHA during March ($P<0.001$). No GnRHA effects were evidenced in June frogs, while in October animals GnRHA was able to increase again the androgen concentration ($P<0.001$). However, the hormone levels achieved during October were significantly lower ($P<0.001$) as compared with those measured in March. Conversely, interstitial area (Fig. 3) of October testes appeared unaffected by GnRHA while June testes showed greater values in peptide treated animals ($P<0.001$). A strong stimulation was achieved in March testes ($P<0.001$) in which the interstitial area increased about 3 fold and reached value significantly higher as compared with June values ($P<0.001$).

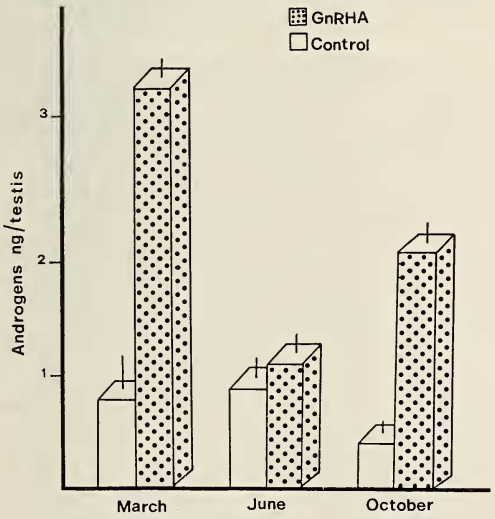

FIG. 2. Testicular androgen concentration of vehicle □ and GnRHA ⊡ treated frogs, *Rana esculenta*. Duncan's test was used to evaluate significance of differences among vehicle or GnRHA-injected animals. Student's "*t*" test was used to evaluate significance of differences between vehicle and GnRHA-injected animals of the same month. Values are the mean ± SD.

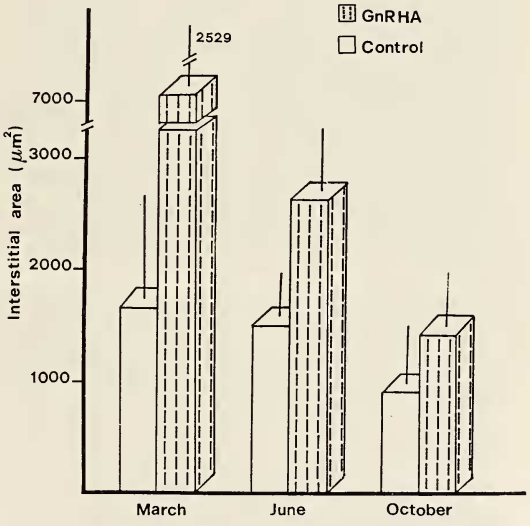

FIG. 3. Intestitial areas calculated in testes of vehicle □ and GnRHA ⊡ treated frogs, *Rana esculenta*. Significance of differences was evaluated as described in Fig. 2. Values are the mean ± SD.

c) *Measurement of spermiation activity in different periods of the year*

GnRHA induced spermiation in all periods examined ($P<0.005$) although the efficiency of the phenomenon showed differences (Table 1). Indeed, March and October testes were highly sensitive to the peptide as compared with June testes ($P<0.005$). Only 40 of 100 tubules examined were empty as compared with near to 100% spermiating

Table 1. Sperm releasing activity of vehicle and GnRHA treated frogs *Rana esculenta*

	MARCH		JUNE		OCTOBER	
CONTROL	+ 0 −100	(a)	+10 −90	(b)	+ 5 − 95	(c)
GnRHA	+ 95 − 5	(d)	+40 −60	(e)	+100 − 0	(f)

a vs d $P<0.05$
b vs e $P<0.05$
c vs f $P<0.05$
d+f vs e $P<0.005$
+ and − represent spermiating and non-spermiating tubules. Spermiation was calculated as the number of empty tubules per 100 total tubules observed in randomly chosen sections. Significance of differences was calculated using χ^2 test.

tubules observed in March and October. It is interesting to note (Fig. 4) that in March testes of control animals almost exclusively spermatozoa were present. Conversely, tubules characterized by the presence of all spermatogenic stages (except spermatozoa, since the presence of spermiation) were observed in GnRHA treated animals. Animals captured in June (Fig. 5) had testes fully organized without differences (control vs GnRHA treated animals) in respect to the development of germinal cells which were all represented. October testes (Fig. 6) presented few spermatocytes and spermatides, while tubules full of spermatozoa, which almost disappeared after GnRHA treatment, were observed.

DISCUSSION

GnRH elicits gonadotropin discharge from the pituitary in vertebrates [1]. In amphibians, the peptide elicits both LH and FSH release whose secretory pattern appears similar in frogs [10] but not in toads [11] where FSH is detectable in plasma when LH is at base-line values. In frogs, gonadotropins act in males, through a low FSH/LH specific receptor [12], primarily stimulating androgen production by Leydig cells [3] and influencing spermatogonial proliferation [13, 14]. GnRHA treatments described here indicate that the testis undergoes a cyclic seasonal responsiveness, by both endocrine and germinal compartments, which occurs separately during the year. Although germinal and interstitial compartments of toad testis may be stimulated separately by FSH and LH, respectively [11], this does not seem to be the case in frogs [10]. Present data in the frog, *Rana esculenta,* show that maximal androgen production is available in March, while germinal compartment appears to respond to a greater extent during spring-early autumn period. The measurement of the interstitial area shows that the steroidogenic compartment becomes highly developed during the period of maximal androgen production (March). Interstitial area is still stimulated during June when androgen production is not affected by GnRHA. Moreover, in October the GnRHA treatment induces androgen production, although to a less extent as compared with March, but interstitial area remains unaffected. This may indicate that the steroidogenic response to the GnRHA treatment is in a certain degree independent of the trophic response by the interstitial compartment. Substances other than androgens may be produced by the interstitium under gonadotropin stimulation to support the gonadal activity, as suggested in mammals [15]. Of course, the knowledge of FSH and LH profiles would be helpful in elucidating the problem. Unfortunately, frog antisera currently available do not crossreact with *Rana esculenta* pituitary homogenate (Licht and Pierantoni, unpublished). Maximal responsiveness of the steroidogenic tissue appears to occur concomitantly with the androgen peak which has been detected in March during the annual cycle, while lack of stimulation of androgen production has been evidenced in June when plasma

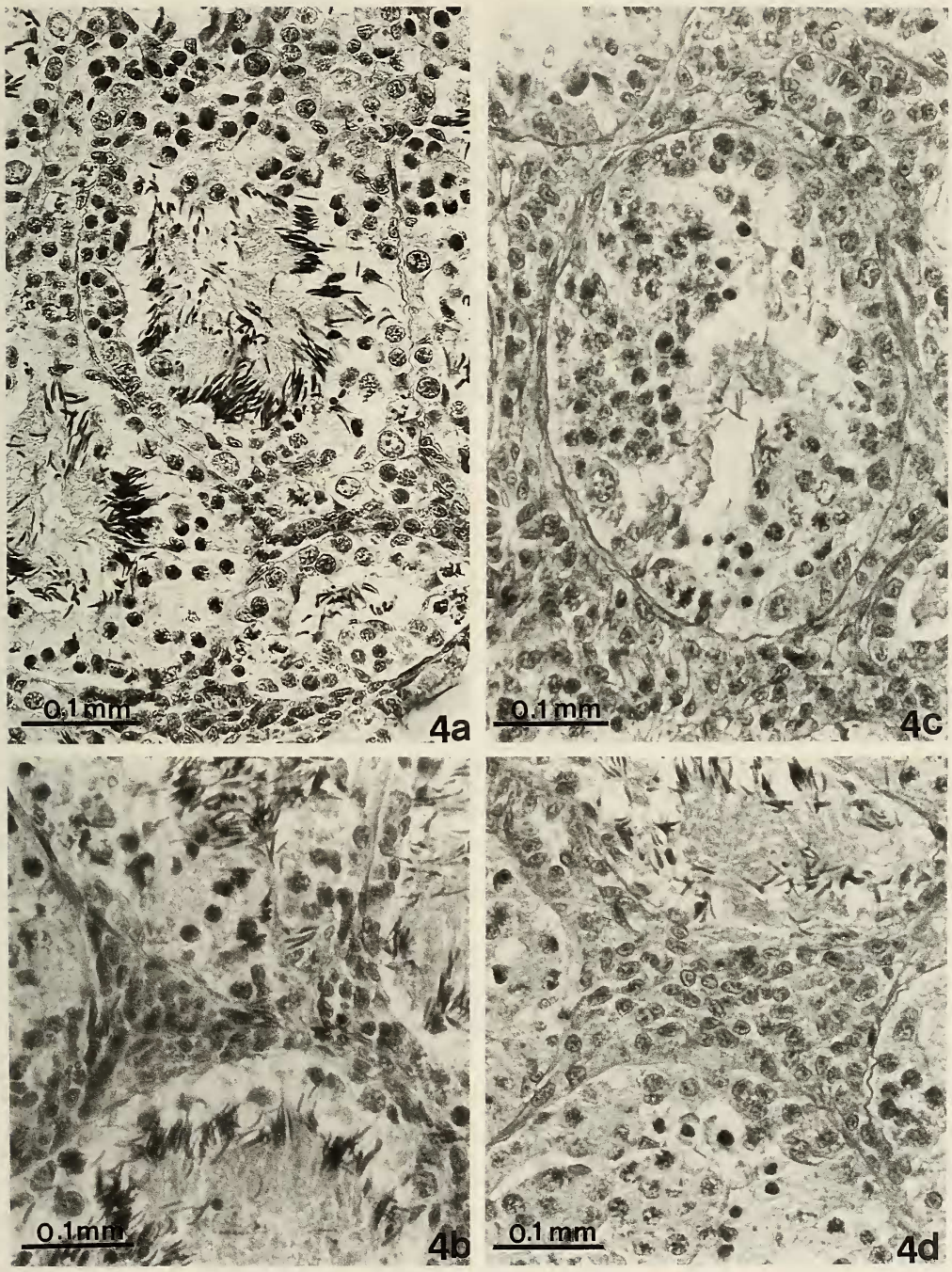

FIG. 4. Testes of March animals treated with vehicle characterized by a) presence of numerous spermatozoa and b) normal interstitial tissue. GnRHA-treated frogs show c) absence of spermatozoa and d) abundant interstitial tissue.

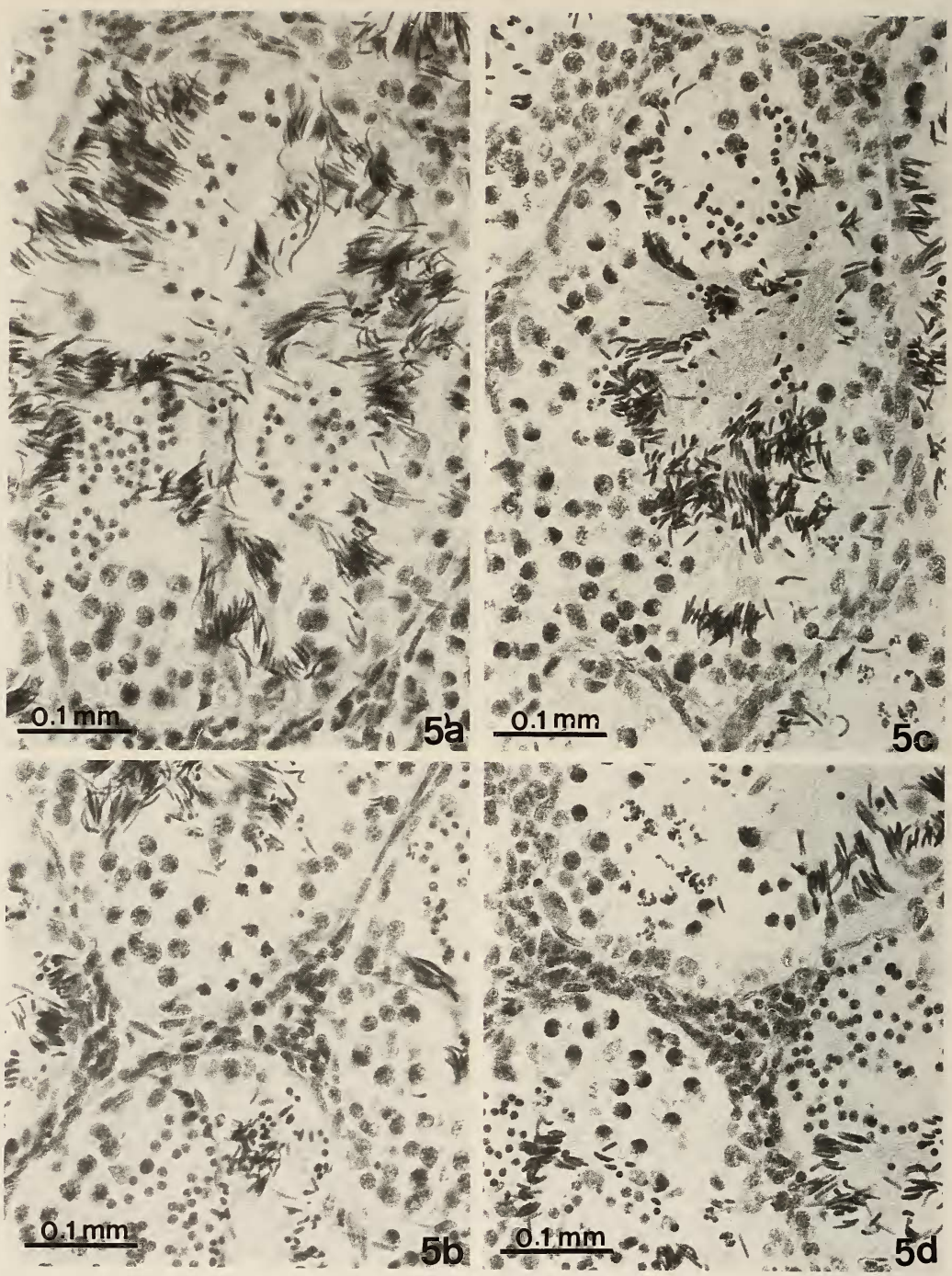

Fig. 5. Testes of June animals treated with vehicle (a and b) and GnRHA (c and d). Germinal compartment is fully organized and spermatozoa are still present after GnRHA treatment.

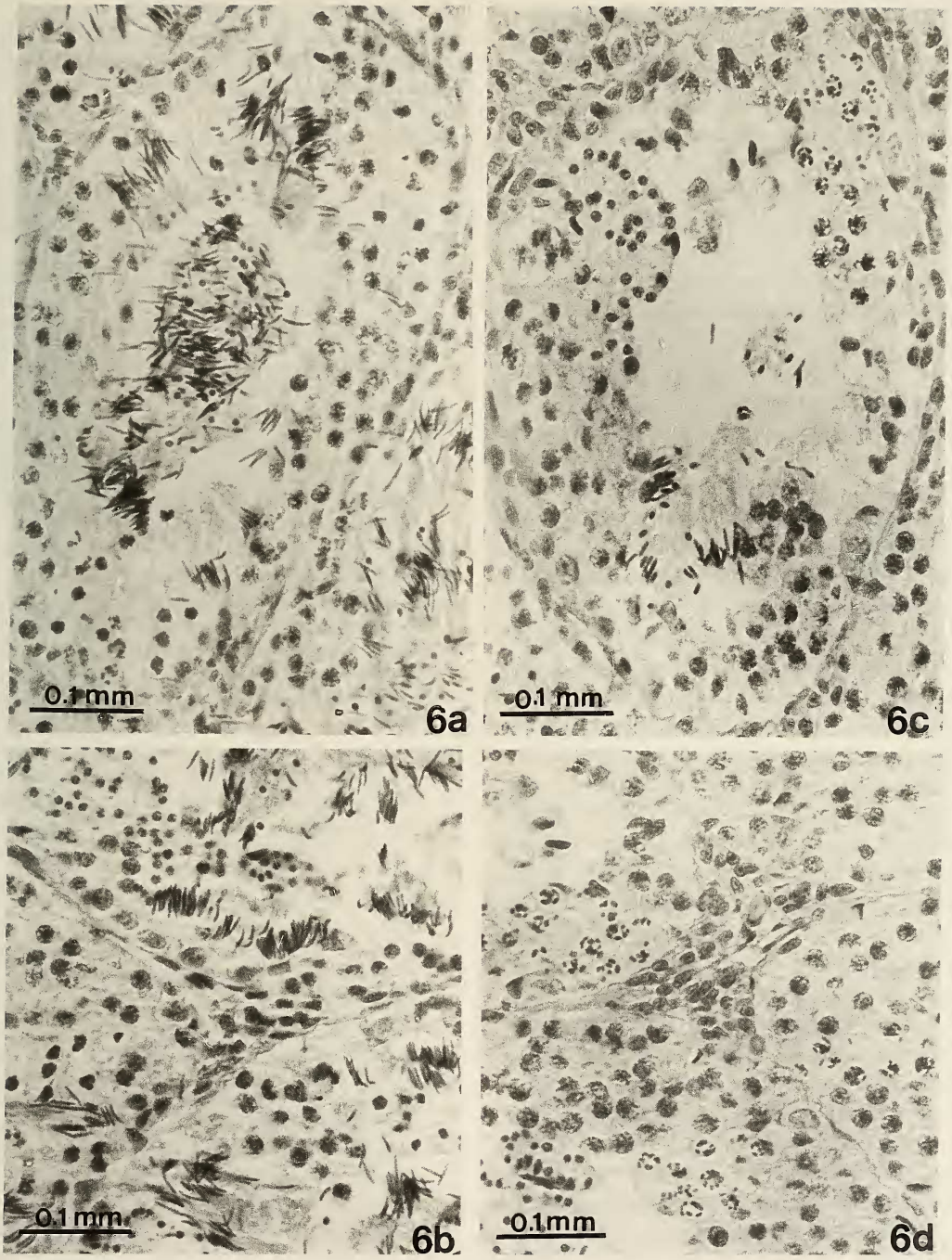

FIG. 6. Testes of October animals treated with vehicle a) characterized mostly by the presence of few spermatocytes, spermatides and spermatozoa and b) by regressed interstitial compartment. GnRHA-treated frogs show c) absence of spermatozoa and d) interstitial compartment development similar to that of vehicle treated animals.

and testicular androgen levels reach the nadir [4, 6, 7, 8]. This *in vivo* experiment confirms previous *in vitro* results showing that minced testes do not respond to any stimulus during summer in term of androgen production [4]. Therefore, our data strongly suggest that intratesticular mechanisms may determine an endocrine refractoriness. On the contrary, spermatogonial proliferation can be stimulated at any time, although at different extent. Spermatogonial proliferation occurs, in *Rana esculenta,* all around the year [16] and reaches maximal values, as measured by mitotic index, during April and October. Moreover, spermatogenic wave can be stimulated by appropriate hormonal and environmental factors (light and temperature) either during winter or during summer [17]. Our results confirm the basal profile of I SPG proliferation and indicate that the germinal compartment is more responsive to GnRHA treatment during summer-early autumn, concomitantly with the appearance of the maximal rate of the spermatogenic wave [18].

As for the sperm-releasing activity, spermiation has been detected in all period studied, and comparable sperm releasing activity occurs in March and October. An interesting observation to emerge is that the high rate of spermiation does not indicate the existence of a similar organization of the germinal compartment. Indeed, March testis appears to be different from October testis as far as the germinal compartment composition it concerns. Despite the diverse histological picture, spermination, after GnRHA treatment, is strong and comparable between the two periods. A cycle of responsiveness is also evident since June testes, although these are characterized by the presence of all spermatogenic stages, appear to respond with minor efficiency.

In conclusion, testes of the frog, *Rana esculenta* show cycle of responsiveness to GnRHA treatment in term of androgen production, interstitial growth, I SPG multiplication and spermiation. All these events are not concomitant but each of them is peculiar of the period of the year in which the experiment is carried out. This may be ascribed to internal testicular factors which determine how the gonads respond or utilize the gonadotropin stimulation.

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